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Genome-Wide Association Study Reveals Variants in *CFH* and *CFHR4* Associated with Systemic Complement Activation

Implications in Age-Related Macular Degeneration

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Purpose: To identify genetic variants associated with complement activation, which may help to select age-related macular degeneration (AMD) patients for complement-inhibiting therapies.

Design: Genome-wide association study (GWAS) followed by replication and meta-analysis.

Participants: AMD patients and controls (n = 2245).

Methods: A GWAS on serum C3d-to-C3 ratio was performed in 1548 AMD patients and controls. For replication and meta-analysis, 697 additional individuals were genotyped. A model for complement activation including genetic and non-genetic factors was built, and the variance explained was estimated. Haplotype analysis was performed for 8 SNPs across the *CFH/CFHR* locus. Association with AMD was performed for the variants and haplotypes found to influence complement activation.

Main Outcome Measures: Normalized C3d/C3 ratio as a measure of systemic complement activation.

Results: Complement activation was associated independently with rs3753396 located in *CFH* ($P_{discovery} = 1.09 \times 10^{-15}$; $P_{meta} = 3.66 \times 10^{-21}$; $\beta = 0.141$; standard error [SE] = 0.015) and rs6685931 located in *CFHR4* ($P_{discovery} = 8.18 \times 10^{-7}$; $P_{meta} = 6.32 \times 10^{-8}$; $\beta = 0.054$; SE = 0.010). A model including age, AMD disease status, body mass index, triglycerides, rs3753396, rs6685931, and previously identified SNPs explained 18.7% of the variability in complement activation. Haplotype analysis revealed 3 haplotypes (H1−2 and H6 containing rs6685931 and H3 containing rs3753396) associated with complement activation. Haplotypes H3 and H6 conferred stronger effects on complement activation compared with the single variants ($P = 2.53 \times 10^{-14}$; $\beta = 0.183$; SE = 0.024; and $P = 4.28 \times 10^{-4}$; $\beta = 0.144$; SE = 0.041; respectively). Association analyses with AMD revealed that SNP rs6685931 and haplotype H1−2 containing rs6685931 were associated with a risk for AMD development, whereas SNP rs3753396 and haplotypes H3 and H6 were not.

Conclusions: The SNP rs3753396 in *CFH* and SNP rs6685931 in *CFHR4* are associated with systemic complement activation levels. The SNP rs6685931 in *CFHR4* and its linked haplotype H1−2 also conferred a risk for AMD development, and therefore could be used to identify AMD patients who would benefit most from complement-inhibiting therapies. *Ophthalmology 2018;125:1064-1074*

See Editorial on page 962.

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The complement system is an integral part of our innate immunity. Its best known physiologic functions are host defense against foreign intruders and homeostasis maintenance.[1] It consists of more than 30 plasma proteins and cellular components that interact in proteolytic cascades for an efficient and rapid activation leading to inflammation, opsonization, and targeted cytolysis.[2] The complement system can be activated by 3 different pathways: the classical pathway, the lectin pathway, and the alternative pathway (AP). The classical pathway is activated by antibody−antigen complexes and the lectin pathway is activated by lectin or ficolin binding to carbohydrates, both on the surfaces of pathogens. In contrast, the AP is activated constitutively at a low level in a process known as tick-over.[3]

All 3 pathways lead to the formation of complement component 3 (C3) convertases that catalyze a proteolytic cleavage of complement C3 into the potent anaphylatoxin

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C3a, and C3b, an opsonization molecule that can be further cleaved into C3d. Complement component 3b also can bind the cleaved form of factor B (Bb) to form the AP C3 convertase (C3bBb) that will cleave more C3, initiating an amplification loop. Downstream in the cascade, complement component 5 convertases are formed, initiating the terminal pathway with the subsequent formation of additional activation products as well as the membrane-attack complex that is responsible for cytolysis.[4] The complement system can be amplified rapidly, and therefore several inhibitory proteins such as complement factor H (FH) and complement factor I are in place regulating complement activity.[4]

Deregulation and deficiencies of the complement system have been reported to be associated with numerous inflammatory, autoimmune, neurodegenerative, and infectious disorders.[5] A prime example of a multifactorial disease associated with a deregulation of the complement system is age-related macular degeneration (AMD). Age-related macular degeneration is characterized by a progressive degeneration of the central retina and is responsible for most cases of vision loss in the elderly with a pooled prevalence of 8.9%.[6,7] Age-related macular degeneration constitutes a major health problem as by 2020, the number of people affected by a form of this disease is projected to be 196 million, rising to 288 million by 2040.[8] Several lines of evidence point toward an overactivation of the complement system in AMD, mainly through a dysregulation of the AP. Multiple genetic variants in or near complement genes (*CFH*, *C3*, *CFI*, *C2/CFB* locus, and *C9*) have been associated strongly with AMD.[9,10] Moreover, complement components have been described in drusen, the hallmark of the disease,[11–14] and complement activation fragments in plasma or serum such as Ba, C3a, C3d, and component C5a have been found to be elevated significantly in AMD patients compared with controls.[15–21] Currently, there is no treatment available for most forms of AMD, nor is there an effective means to halt AMD progression. Therefore, therapies for AMD, as well as for other diseases involving complement deregulation, are being developed aiming to inhibit or lower complement activation.[22–24]

Systemic complement activation levels demonstrate considerable variation among individuals.[16–20] As a consequence, patients who have higher levels of complement activation may benefit more than others from the upcoming therapies. A better understanding of the factors that influence complement activation would facilitate the selection of the most suitable patients for complement-inhibiting therapies. Genetic markers are robust biomarkers that could be included in prediction models for complement activation. Several studies have previously evaluated the effect of genetic variation on complement activity; however, these studies were restricted to a limited number of single nucleotide polymorphisms (SNPs).[16–19,21,25]

The aim of this study was to perform the first genome-wide association study (GWAS) on systemic complement activation levels. Identification of genetic variants explaining complement activation levels will contribute to a better understanding of the molecular mechanisms of complement-related diseases, will pinpoint potential drug targets, and will facilitate the selection of patients for complement-inhibiting therapies.

Methods

Study Population

This study included 2245 participants from the European Genetic Database (www.eugenda.org). The European Genetic Database is a multicenter database for the clinical and molecular analysis of AMD collected at the Radboud University medical center, Nijmegen, The Netherlands, and at the University Hospital of Cologne, Cologne, Germany. The study participants were separated into 2 cohorts: a discovery cohort comprising 1548 individuals and a replication cohort of 697 individuals.

The study was performed in accordance with the tenets of the Declaration of Helsinki (seventh revision) and the Medical Research Involving Human Subjects Act. Approval of the local ethics committee of both University hospitals was obtained, and written informed consent was acquired from all participants. All the individuals included in the study agreed to the performed serum measurements and genotyping. All participants were of European descent and older than 50 years. Age-related macular degeneration and control status were assigned by multimodal image grading according to the standard protocol of the Cologne Image Reading Center by certified graders. Age, sex, height, and weight measurements were obtained by standardized interviewer-assisted questionnaires.

Serum Complement and Lipid Measurements

Serum was obtained by a standard coagulation and centrifugation protocol, and within 1 hour after collection, the samples were stored at −80°C. Triglycerides and high-density lipoprotein cholesterol were measured using standard procedures by a clinical chemistry laboratory (Architect Analyzer; Abbott Diagnostics, Hoofddorp, The Netherlands). Complement component 3 was assessed by radial immunodiffusion (or Mancini method) using monospecific polyclonal rabbit antisera, and C3d was measured by rocket electrophoresis, as previously described.[21] Complement component 3d is a fragment of C3 generated upon activation of the system, and therefore a direct measurement of complement turnover.[4] Moreover, C3d has the longest half-life of all C3 split products.[26] The C3d-to-C3 ratio is a sensitive way of assessing the activation of the complement system independently of the baseline individual C3 concentration.[27–29] The C3d-to-C3 ratio has been described previously to be a robust biomarker for complement activation in AMD studies.[19] The different measurements were performed for all samples in a single assay.

Genotyping

Genomic DNA was extracted from peripheral blood samples using standard procedures. The discovery cohort was genotyped with a custom-designed HumanCoreExome array by Illumina (Illumina Inc., San Diego, CA) within the International AMD Genetics Consortium. All the details regarding the design of the array, annotation, imputation, and quality control of the genotypic data have been described previously.[9]

Imputed lead variants in GWAS peaks that reached significance, rs6685931 and rs3130572, were confirmed by polymerase chain reaction and Sanger sequencing. The SNP rs6685931 was evaluated in 12 individuals representing the 3 genotypes, and a 100% of concordance with the imputed genotypes was achieved. The SNP rs3130572 (chromosome 6) was located in a highly

repetitive region and specific primers could not be designed; therefore, this SNP was excluded from further analysis. In the replication cohort, *CFH* rs3753396 and *CFHR4* rs6685931 were genotyped using competitive allele-specific polymerase chain reaction assays according to the manufacturer instructions (KASP Genotyping Chemistry; LGC, Hoddesdon, UK).

Statistical Analysis

Natural log transformation was applied to normalize the skewed distribution of C3d/C3 measurements. A general linear model for ln(C3d/C3) including as independent variables the environmental factors collected was used to determine potential confounders. The R^2 and adjusted R^2 statistics were estimated for the model. Additionally, the R^2 statistic was estimated for each of the independent factors individually, performing separate models. Analyses were carried out using SPSS software version 20.0 (IBM Software and Systems, Armonk, NY).

A power calculation for the GWAS was performed using the Genetic power calculator.[30] Association tests in the GWAS and replication analyses were performed by means of a linear Wald test from EPACTS software (http://genome.sph.umich.edu/wiki/EPACTS) using allele dosages. Linear regression models adjusted for age, sex, body mass index (BMI), triglycerides, clinic site, and the first 2 ancestry principal components were used. Manhattan and Q-Q plots were generated using the 'qqman' R package (version 0.1.2; R Foundation for Statistical Computing, Vienna, Austria). The regional plots for chromosome 1 were generated using LocusZoom.[31] Meta-analysis of fixed effects based on effect size estimates and standard errors was performed using METAL software (version 2-11-03-25).[32]

Evaluation of an interaction between the identified SNPs and clinic or AMD status was performed including an interaction parameter on the general linear model and assessing nominal significance. Comparisons of systemic complement activation levels between the genotype groups were performed using a general linear model adjusted for age, BMI, triglycerides, and clinic sites including both the discovery and the replication cohorts. SPSS software version 20.0 (IBM Software and Systems, Armonk, NY) was used for these analyses.

To estimate how much of the variation in systemic complement activation could be explained by the identified factors, general linear models for systemic complement activation were performed using SPSS software version 20.0. Only the 1548 individuals from the discovery cohort were included to accommodate the *CFH* rs800292 and *C2* rs9332739 SNPs, which were not analyzed in the replication cohort. The adjusted R^2 statistic was estimated for the models.

Haplotype analysis was carried out for the 1548 patients genotyped with exome-arrays using the haplo.glm function of the R library 'haplo.stats' (version 1.7.7). Analysis was performed based on a general linear model adjusted for age, sex, BMI, triglycerides, clinic site, and the first 2 ancestry principal components.

Single-variant and haplotype association analyses with AMD were performed for the 1548 individuals of the discovery cohort. Single-variant analyses were performed using a Firth bias-corrected likelihood-ratio test with EPACTS software. Haplotype analyses were based on chi-squared tests including haplotypes with a predicted probability of 0.75 or more using SPSS software version 20.0.

Risk scores for AMD-associated variants were calculated as a sum of the number of AMD risk-increasing alleles. Two risk scores were calculated: the first risk score included the 52 AMD-associated variants described in Fritsche et al,[9] and the second risk score included the 19 variants located in or near complement genes of these 52. The variants included in the complement risk score were: rs10922109, rs570618, rs121913059, rs148553336, rs187328863, rs61818925, rs35292876, and rs191281603 from the *CFH* locus; rs10033900 and rs141853578 from the *CFI* locus; rs62358361 from the *C9* locus; rs116503776, rs144629244, rs114254831, and rs181705462 from the *C2/CFB/SKIV2L* locus; rs11080055 from the *TMEM97/VTN* locus; and rs2230199, rs147859257, and rs12019136 from the C3 locus. The risk scores were included in linear models for ln(C3d/C3) that included age, BMI, triglycerides, and clinic site as covariates, and the effect of the risk score was estimated. The 1548 individuals from the discovery phase, genotyped with the HumanCoreExome array, were included in these analyses. Figures including graphs were generated using Graphpad Prism version 5.03 (GraphPad Software, La Jolla, CA).

Results

Characteristics of the Study Cohorts

We evaluated the association of genetic variants with systemic complement activation levels through a GWAS in a discovery cohort of 1548 individuals, followed by replication in an independent cohort of 697 individuals. For both cohorts, demographics and information about AMD disease status, BMI, triglycerides, and high-density lipoprotein cholesterol was collected (Table 1).

Higher complement activation levels were associated independently with older age, AMD disease status, lower BMI, and lower triglyceride levels as previously described.[21,33] Differences also were observed between the sample collection clinics (Table S2, available at www.aaojournal.org). Therefore, these factors were included as covariates in all consecutive analyses.

Genome-Wide Association Study Identifies 2 Independent Signals at the *CFH/CFHR* Locus to Be Associated with Systemic Complement Activation

We carried out a GWAS of normalized C3d/C3 levels as a measure of systemic complement activation. After quality control, a total of 1548 individuals and 9 972 920 variants were included in the analysis. The study had more than 80% of power to detect common variants (minor allele frequency $\geq$5%), explaining $\geq$2.6% or more of variance in complement activation levels.

A total of 280 variants reached genome-wide significance (Manhattan plot, Fig 1A; QQplot, Fig S2, available at www.aaojournal.org; $\lambda_{GC} = 0.999$). All variants, except for one, were located on chromosome 1 at the *CFH/CFHR* locus (chromosome 1, 196.643.724−197.061.086). The only variant outside of this locus was located on chromosome 6 near the *PSORS1C1* gene, but could not be verified by Sanger sequencing. The SNP rs3753396 (c.2016A→G, p.Gln672Gln) located in exon 14 of the complement factor H (*CFH*) gene showed the strongest association with complement activation levels ($P = 1.09 \times 10^{-15}$; $\beta = 0.145$; standard error [SE], 0.018; Table 3; locus zoom depicted in Fig 1B).

Conditional analysis on the lead SNP revealed a second independent signal with a P value close to genome-wide significance for which the strongest associated variant was rs6685931. This SNP was also located at the *CFH/CFHR* locus, specifically in intron 1 (c.59−4315T→C) of the complement factor H related 4 (*CFHR4*) gene ($P = 8.18 \times 10^{-7}$; $\beta = 0.068$; SE, 0.014; Table 3; locus zoom depicted in Fig 1C).

Table 1. Demographics and Other Characteristics of the Discovery and Replication Cohorts

	Discovery Cohort (n = 1548)	Replication Cohort (n = 697)
Complement activation ln(c3d/c3), mean (SD)	1.459 (0.407)	1.464 (0.398)
Age (yrs), mean (SD)	73.2 (7.8)	73.3 (7.7)
Female sex (%)	60	58.8
AMD disease status, control (%)	53.7	37.4
BMI (kg/m^2), median (quartiles)	25 (23–28)	25 (23–28)
Triglycerides (mmol/l), median (quartiles)	1.620 (1.170–2.220)	1.620 (1.165–2.210)
HDL cholesterol (mmol/l), mean (SD)	1.489 (0.377)	1.478 (0.403)
Clinic site - Radboud university medical center (%)	53.5	63

AMD = age-related macular degeneration; BMI = body mass index; HDL = high-density lipoprotein; SD = standard deviation.

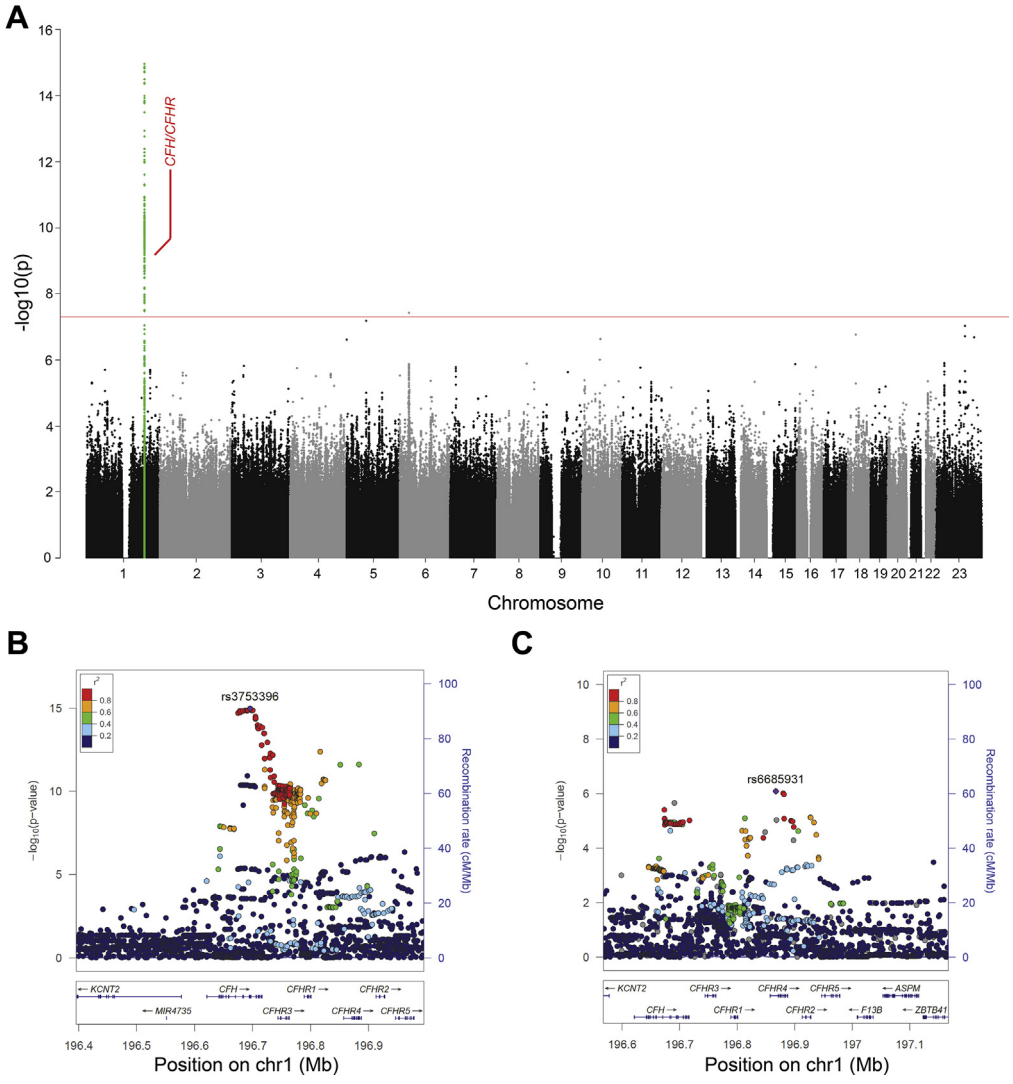

Figure 1. Graphs showing that the genome-wide association study identified 2 independent signals at the *CFH/CFHR* locus associated with systemic complement activation levels. **A**, Manhattan plot illustrating the *P* values of each individual single nucleotide polymorphism (SNP) tested for association with systemic complement activation. The red horizontal line indicates the threshold considered for genome-wide significance ($P = 5 \times 10^{-8}$). **B**, Locus zoom plot showing a detailed view of the chromosome 1 signal. The lead SNP rs3753396 is located in the *CFH* gene. The SNPs are colored based on their linkage disequilibrium estimate (r^2) to the lead SNP. **C**, Locus zoom plot showing a detailed view of the signal on chromosome 1 (chr1) after conditioning the association analysis for rs3753396. Here, the lead SNP rs6685931 is located in the *CFHR4* gene. The SNPs are colored based on their linkage disequilibrium estimate (r^2) to the lead SNP.

Table 3. Meta-analysis of Discovery and Replication Cohorts Identifies 2 Signals at the *CFH/CFHR* Locus Associated with Systemic Complement Activation Levels

Lead Variant (Minor Allele)	Imputation Quality (Rsq)*	Chromosome Position†	Gene‡	Discovery Cohort (n = 1548)			Replication Cohort (n = 697)§			Meta-analysis (n = 2245)¶	
				Minor Allele Frequency	*β (Standard Error)*	*P Value*	*Minor Allele Frequency*	*β (Standard Error)*	*P Value*	*β (Standard Error)*	*P Value*
rs3753396 (G)	—	1:196 695 742	*CFH*	0.168	0.145 (0.018)	1.091×10^{-15}	0.147	0.131 (0.027)	1.390×10^{-6}	0.141 (0.015)	3.664×10^{-21}
rs6685931 (C)	0.99	1:196 867 233	*CFHR4*	0.439	0.068 (0.014)	8.184×10^{-7}	0.493	0.038 (0.014)	8.620×10^{-3}	0.054 (0.010)	6.320×10^{-8}

*Not applicable for genotyped variants (—).
†Chromosome and chromosomal positions described according to the reference sequence database of the National Center for Biotechnology Information (NCBI RefSeq) hg19 human genome.
‡Closest gene to the lead variant.
§Replication cohort for rs6685931 consisted of 686 individuals.
¶Meta-analysis for rs6685931 was performed in a total of 2234 individuals.

Variants shown to be associated with complement activation fragments in previous studies were extracted from the GWAS results.[17,18] The SNP rs800292 in *CFH* and the 2 SNPs in linkage disequilibrium rs4151667 in *CFB* and rs9332739 in *C2* were associated nominally with systemic complement activation levels in the current study, showing the same direction of the effect. The SNP rs2230199 in *C3* and the SNP rs10490924 in *ARMS2* could not be replicated (Table S4, available at www.aaojournal.org).

Replication in an Independent Cohort Confirms the Effect of rs3753396 in *CFH* and rs6685931 in *CFHR4* on Systemic Complement Activation

Replication analysis of rs3753396 in *CFH* and rs6685931 in *CFHR4* in an independent cohort of 697 study participants confirmed both variants to be associated significantly with systemic complement activation levels (rs3753396: $P = 1.39 \times 10^{-6}$; $\beta = 0.131$; SE, 0.027; and rs6685931: $P = 8.62 \times 10^{-3}$; $\beta = 0.038$; SE, 0.014; Table 3). Subsequent meta-analysis showed associations for both rs3753396 ($P = 3.66 \times 10^{-21}$; $\beta = 0.141$; SE, 0.015) and rs6685931 ($P = 6.32 \times 10^{-8}$; $\beta = 0.054$; SE, 0.010), confirming that 2 independent signals at the *CFH/CFHR* locus are associated with higher complement activation levels (Table 3). Sensitivity analyses adjusting for AMD disease status showed comparable results (Table S5, available at www.aaojournal.org), and neither an interaction between clinic site and the identified SNPs ($P_{rs3753396 \times clinic} = 0.436$; $P_{rs6685931 \times clinic} = 0.676$), nor an interaction between AMD status and the identified SNPs ($P_{rs3753396 \times AMD\ status} = 0.557$; $P_{rs6685931 \times AMD\ status} = 0.658$) was detected.

Next, mean complement activation levels in the genotype groups of rs373396 and rs6685931 were analyzed. For rs3753396 in *CFH*, the heterozygous AG genotype group showed higher complement activation levels compared with the reference AA genotype group ($P = 6.23 \times 10^{-18}$; $\beta = 0.152$; SE, 0.018), and for the homozygous GG group, these levels were even higher ($P = 2.39 \times 10^{-7}$; $\beta = 0.267$; SE, 0.052; Fig 3A). In the case of rs6685931 in *CFHR4*, a similar effect was observed: the heterozygous TC genotype group had higher complement activation levels than the reference TT genotype ($P = 10^{-3}$; $\beta = 0.063$; SE, 0.019) and for the homozygous CC group, the levels were even higher ($P = 3.62 \times 10^{-7}$; $\beta = 0.118$; SE, 0.023; Fig 3B). Analysis of the cumulative effect of both SNPs showed that the main effect on systemic complement activation levels is driven by rs3753396 in *CFH*, and rs6685931 in *CFHR4* introduces additional variation to the rs3753396 genotypes (Fig 3C).

A Model of Genetic and Nongenetic Variables Explains 18.7% of the Variability in Complement Activation

General linear models were built to determine how much of the variation could be explained by factors found to be associated with systemic complement activation. A model including only nongenetic factors (age, AMD disease status, BMI, and triglycerides) explained 12.6% of the variability in systemic complement activation. With the addition of SNP rs3753396 to the model, 16.3% of the variability could be explained, and by including SNP rs6685931, a total of 17.3% was explained. We additionally incorporated SNPs associated with complement activation fragments in a previous study that replicated in our GWAS: rs800292 in *CFH* and rs9332739 in *C2*.[18] Only rs9332739 remained associated independently with systemic complement activation levels, and the variance explained by the model rose to 18.7% (adjusted R^2; Table 6).

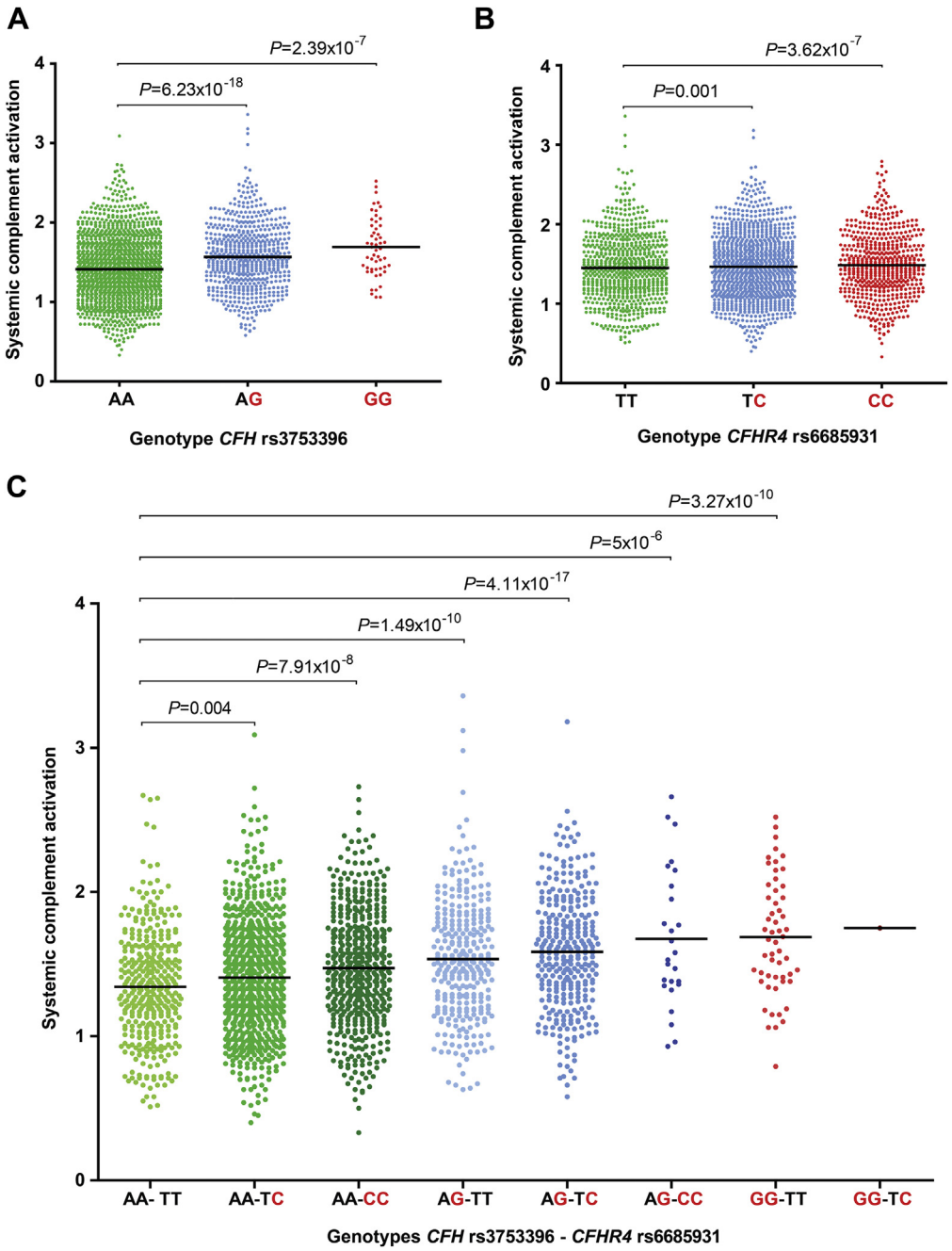

Figure 3. Graphs showing systemic complement activation levels stratified by rs3753396 and rs6685931 genotypes: rs6685931 introduced additional variation on the main effect of rs3753396. The y-axes represent the ln-transformed complement C3d/C3 ratio as a measure of systemic complement activation. Horizontal bars indicate the mean values for each genotype group. The complement-raising alleles for both single nucleotide polymorphisms are indicated in red. Association analyses included the 2245 individuals from the discovery and the replication cohorts. **A**, Distribution of complement activation levels for each genotype of rs3753396 in *CFH*. **B**, Distribution of complement activation levels for each genotype of rs6685931 in *CFHR4*. *P* values were calculated adjusting the model for rs3753396. **C**, Distribution of complement activation levels over the genotype combinations of rs3753396 in *CFH* and rs6685931 in *CFHR4*.

Haplotypes across the *CFH/CFHR* Locus Show Stronger Effects on Systemic Complement Activation Levels Compared with Individual Variants

To assess whether more variants at the *CFH/CFHR* locus influence systemic complement activation and to determine the cumulative effect of several variants on the same haplotype, we evaluated the effect of distinct haplotypes across the *CFH/CFHR* locus on systemic complement activation. Haplotypes previously described for AMD already included rs3753396, the lead variant associated in the GWAS,[34] and were expanded by adding rs668593, the second independent signal. In total, 7 SNPs across the *CFH/CFHR* locus yielded 9 different haplotypes with a predicted population

Table 6. Model of Genetic and Nongenetic Variables Explaining 18.7% of the Variability in Systemic Complement Activation

	β	Standard Error (β)	P Value
CFH rs3753396			
AG	0.196	0.023	8.772×10^{-17}
GG	0.330	0.066	6.461×10^{-7}
CFHR4 rs6685931			
TC	0.070	0.024	0.003
CC	0.125	0.033	1.620×10^{-4}
CFH rs800292			
GA	−0.011	0.023	0.639
AA	0.027	0.046	0.555
C2 rs9332739			
GC	−0.185	0.034	4.674×10^{-8}
CC	0.168	0.213	0.431
Age (yrs)	0.004	0.001	0.005
Disease status (AMD)	0.035	0.020	0.089
BMI (kg/m^2)	−0.012	0.003	2×10^{-6}
Triglycerides (mmol/l)	−0.131	0.011	1.177×10^{-33}

AMD = age-related macular degeneration; BMI = body mass index.
$R^2 = 0.193$ (adjusted $R^2 = 0.187$). The model included the 1548 individuals from the discovery phase.

frequency higher than 1% (Table 7; Table S8, available at www.aaojournal.org).

Association with systemic complement activation levels revealed haplotypes with stronger effects on complement activation compared with the single SNPs identified in the GWAS. Haplotypes H1−2, H3, and H6 were associated with higher systemic complement activation levels. Haplotype H3 carrying the complement-raising allele of rs3753396 (G) had a stronger effect on complement activation levels ($P = 2.53 \times 10^{-14}$; β = 0.183; SE, 0.024) compared with the complement-raising allele of rs3753396 in the single variant analysis (β = 0.141; SE, 0.015). Haplotypes H1−2 and H6 both carried the complement-raising allele for rs6685931 (C). Haplotype H6 showed a stronger effect on complement activation levels ($P = 4.82 \times 10^{-4}$; β = 0.144; SE, 0.041) compared with the single variant analysis for rs6685931 (β = 0.054; SE, 0.010; Table 7; Table S8, available at www.aaojournal.org).

The Single Nucleotide Polymorphism rs6685931 in *CFHR4* and Haplotype H1−2 Confer a Risk for Age-Related Macular Degeneration

To identify genetic biomarkers that are relevant in the context of disease, we explored whether the SNPs and haplotypes associated with systemic complement activation levels also associate with AMD. The SNP rs3753396 in *CFH* was not associated with AMD ($P = 0.76$). In contrast, the complement-raising allele of rs6685931 in *CFHR4* (C) was associated with an increased risk for AMD ($P = 5.89 \times 10^{-12}$; odds ratio = 1.631; 95% confidence interval, 1.489−1.772; Table 9). These results are in concordance with the largest GWAS on AMD reported to date (rs3753396, $P = 3 \times 10^{-3}$; rs6685931, $P = 1.02 \times 10^{-495}$; odds ratio >1).[9]

In agreement with the single variant analysis of *CFH* rs3753396, the haplotype H3 that gave the highest risk for higher systemic complement activation was not associated with AMD ($P = 0.80$). Haplotype H6 carries the *CFHR4* rs6685931 complement-raising allele (C), but did not reach significance in the association with AMD ($P = 0.14$); however, the frequency of H6 was relatively low (3%). Haplotype H1−2, the most common haplotype carrying the *CFHR4* rs6685931 complement-raising allele (C), showed a strong risk-conferring association with AMD ($P = 1.38 \times 10^{-12}$; odds ratio = 1.318; 95% confidence interval, 1.223−1.420; Table 9; Fig 4).

Finally, we determined whether other AMD-associated variants are associated with systemic complement activation levels. For this purpose, we extracted the 52 AMD-associated variants reported in the largest AMD study performed so far from the GWAS on complement activation levels.[9] However, no variants outside of the *CFH/CFHR* locus were found to be associated with systemic complement activation levels at the genome-wide significance level, or at a significance level of $P < 0.05/52 = 0.001$ (Table S10, available at www.aaojournal.org). Interestingly, a risk score based on the 52 AMD risk-conferring alleles was associated with higher levels of complement activation ($P = 0.043$; β = 0.004; SE(β) = 0.002). A similar risk score including only the variants located in or near complement genes was associated more strongly with higher levels of complement activation ($P = 0.022$; β = 0.009; SE(β) = 0.004). This complement risk score included 3 nominally associated variants: 2 common variants located in the *CFH* and the *C2/CFB/SKIV2L* loci—rs10922109 and rs116503776, respectively—and a rare variant located in the *CFI* gene, rs141853578 or p.Gly119Arg. However, the effects of these genetic risk scores are smaller

Table 7. Association of Haplotypes across the *CFH/CFHR* Locus with Systemic Complement Activation Levels

Haplotype	*CFH* rs3753396 and *CFHR4* rs6685931 Alleles	Haplotype Frequency	β	Standard Error (β)	P Value
H2	A-T	0.18	Reference	Reference	Reference
H1−2	A-C	0.36	0.062	0.019	1.148×10^{-3}
H3	G-T	0.14	0.183	0.024	2.531×10^{-14}
H4	A-T	0.10	0.013	0.026	0.607
H5	A-T	0.04	−0.058	0.038	0.128
H1−1	A-T	0.03	−0.053	0.043	0.218
H6	A-C	0.03	0.144	0.041	4.823×10^{-4}
H7	A-C	0.03	0.060	0.046	0.192
H8	A-T	0.03	−0.007	0.048	0.890

Haplotype association analyses with age-related macular degeneration were performed for the 1548 individuals in the discovery cohort. Haplotypes are coded as in Hageman et al.[34] If 2 different subhaplotypes based on the extra allele in single nucleotide polymorphism rs6685931 were found, the Hageman haplotypes were recoded as 1 or 2. Alleles associated with higher complement levels are underlined. The reference haplotype was set to the most common haplotype not carrying any complement-raising allele for rs3753396 or rs6685931.

Table 9. Association of Complement-Raising Single Nucleotide Polymorphism and Haplotypes with Age-Related Macular Degeneration: Single Nucleotide Polymorphism rs6685931 and Haplotype H1−2 Confer a Risk for Age-Related Macular Degeneration

	Odds Ratio	Confidence Interval	*P* Value
SNP rs3753396	1.031	0.839−1.223	0.756
SNP rs6685931	1.631	1.489−1.772	5.889×10^{-12}
Haplotype H3	1.015	0.911−1.130	0.795
Haplotype H6	0.828	0.637−1.075	0.135
Haplotype H1−2	1.318	1.223−1.420	1.382×10^{-12}

SNP = single nucleotide polymorphism.
Single variant and haplotype association analyses with age-related macular degeneration were performed for the 1548 individuals from the discovery cohort. Haplotype analyses were based on chi-squared tests that compared the frequency of the analyzed haplotypes in patients versus controls.

compared with the single variant effects in the model for systemic complement activation described in Table 6.

Discussion

We conducted a GWAS on systemic complement activation levels, evaluating for an unbiased approach the genetic risk factors involved in the activation of this essential component of the immune system. We identified and replicated 2 common variants, rs3753396 and rs6685931, that lead to higher systemic complement activation levels independently of age, sex, AMD disease status, triglycerides, and BMI. These 2 variants were included in a model for systemic complement activation, which explained 18.7% of its variability.

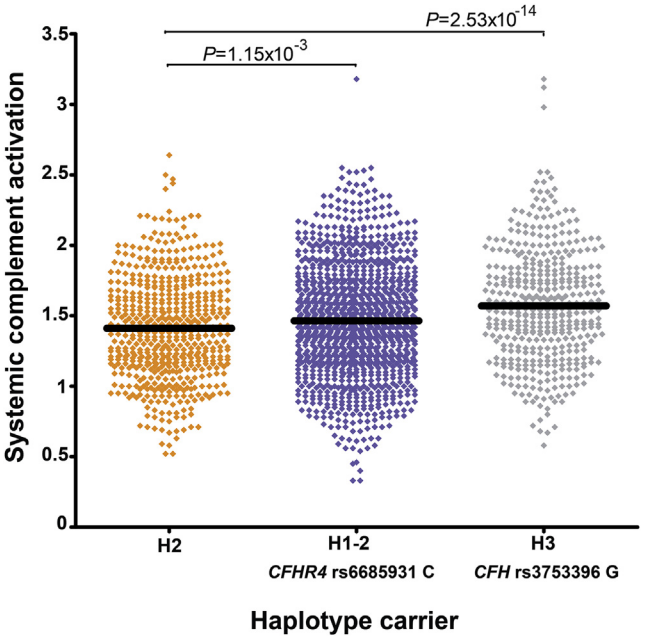

Figure 4. Graph showing complement activation levels stratified by common haplotypes across the *CFH/CFHR* locus. The AMD risk haplotype H1−2 shows high complement activation levels, and the non−AMD-associated H3−1 haplotype shows the highest. Horizontal bars indicate the mean values for each haplotype carrier group. Haplotype carriers included in the graph had a posterior probability higher than 0.75. The haplotype group colors indicate the association with AMD: orange, protective; blue, risk conferring; grey, not associated. Association analyses were carried out for the 1548 patients genotyped with exome array.

The SNP rs3753396 (c.2016A→G, p.Gln672Gln) is a coding, synonymous variant located in exon 14 of the *CFH* gene, and therefore this variant, or the linked causal variant(s), may regulate complement activation levels through FH. Factor H is a key negative regulator of the AP and the amplification loop of the complement cascade, which is expressed constitutively in the liver and locally by other cell types, such as retinal pigment epithelial and endothelial cells.[35−37] Evidence to support the theory that rs3753396 exerts an effect on complement activation through FH comes from genetic studies on other diseases. The SNP rs3753396 has been reported to be associated with atypical hemolytic uremic syndrome, known to be caused by mutations in *CFH*.[38,39] Moreover, reduced susceptibility to meningococcal disease also has been associated with rs3753396. Meningococcal disease is caused by *Neisseria meningitides*, which binds FH to avoid complement-mediated killing.[40] The SNP rs3753396 is in linkage disequilibrium with rs1065489, also located in *CFH* (c.2808G→T, p.Glu936Asp), which was proposed to be the causal variant for meningococcal disease based on in silico pathogenicity predictions.[41]

The SNP rs6685931 (c.59−4315T→C) is located in intron 1 of the *CFHR4* gene. Factor H related 4 (FHR-4) is a glycoprotein that, in contrast to the attenuating effects of FH, seems to promote complement activation. It binds the complement fluid-phase C3b and forms an additional AP C3 convertase (FHR4-C3bBb), which is less susceptible to FH-mediated decay.[42] However, because rs6685931 is in high linkage disequilibrium ($r^2 > 0.8$) with several variants located in the *CFH* gene, either FH or FHR-4 could be responsible for the effects observed on complement activation.

We analyzed the association of genetic variants with systemic complement activation levels in a hypothesis-free manner. The results indicate that with our study design, the genetic variants with the largest effect on complement activation levels are rs3753396 and rs668593, located at the *CFH/CFHR* locus. Moreover, other previously associated variants in *CFH* and *C2/CFB* could be replicated.[18] Haplotype analysis at the *CFH/CFHR* locus revealed 2 haplotypes with stronger effects on complement activation levels compared with the individual SNPs. These findings suggest that additional variants at the *CFH/CFHR* locus play a role in the activation of the complement system. Indeed, several rare coding variants in the *CFH* gene have been shown to lead to increased complement activity.[10] Genetic variants in other genes that influence systemic complement activation levels may be uncovered with larger sample sizes that would allow for the detection of rarer variants and smaller effects. A compelling rare variant candidate that may merit further investigation is *CFI* rs141853578 (p.Gly119Arg), which was found to be nominally significant in our study. This variant has been

associated previously with lower factor I levels in plasma and a lower ability to degrade C3d on the cell surface and C3b in the fluid phase.[43]

In this study, AMD was associated with systemic complement activation, which is in agreement with previous reports.[15–18,20] In our analysis, rs6685931 in *CFHR4* was associated with both systemic complement activation and AMD. Haplotype analyses were in line with these results; we observed that the complement-raising allele of SNP rs6685931 (C) was located mainly on the H1−2 haplotype, which associated with a higher risk for AMD development. Thus, this SNP and its linked haplotype could serve as a robust biomarker for complement activation in the context of AMD and could be used to identify AMD patients who would benefit most from complement-inhibiting therapies.

We noted that the rare haplotype H6 (with a frequency of 3%), also containing rs6685931, had a larger effect on complement activation levels compared with the single variant rs6685931. However, haplotype H6 was not associated significantly with AMD, probably because of statistical power limitations. Studies with larger cohort sizes may clarify the role of the H6 haplotype in AMD and may identify other rare haplotypes that associate with AMD and have larger effects on complement activation levels.

Strikingly, the genetic variant that was associated most strongly with systemic complement activation, rs3753396 in *CFH*, and its main haplotype (H3) did not associate with AMD. However, the SNP rs3753396 and haplotype H3 have been described to confer risk for atypical hemolytic uremic syndrome development. Atypical hemolytic uremic syndrome is a complement system-related disease that leads to systemic thrombotic microangiopathy and renal endothelial injury.[39,44] This finding suggests that the effect of the haplotypes may be different systemically compared with the AMD disease site, possibly through a tissue-specific effect of the genetic variants. Consequently, systemic complement activation may not always reflect complement activation in the disease tissue, and therefore, it may not be the most appropriate measure for AMD studies. Genetic biomarkers such as SNP rs6685931 and haplotype H1−2 are robust markers that, together with the C3d-to-C3 ratio, could serve as biomarkers for complement activity studies in AMD. This is supported by a recent study demonstrating that complement activation levels in aqueous humor are higher than in plasma samples of AMD patients.[45] As a consequence, the effect of rs6685931 and H1−2 on local complement activation may be even larger than the effect seen on systemic levels.

Our results also could further the understanding of other complement-related diseases, as well as be used in the context of personalized medicine involving FH supplementation therapy and other complement-targeting therapies.[46–48] Besides *N. meningitidis*, a number of bacteria, fungi, parasites, and viruses bind FH to avoid elimination by the alternative pathway of the complement system.[49] Also, some cancer cells express FH to avoid being targeted by the immune system.[50–52] Other FH-related diseases for which our results may be of interest include hemolytic uremic syndrome, atypical hemolytic uremic syndrome, encephalomyelitis, atherosclerosis, insulin resistance, immunoglobulin A nephropathy, Alzheimer's disease, cisplatin nephropathy, as well as severe dengue, for which variants in the *CFH* gene have been shown to be protective.[53]

In conclusion, we identified 2 common variants located at the *CFH/CFHR* locus, rs3753396 and rs668593, which strongly influence systemic complement activation levels. Moreover, our haplotype studies suggest that other genetic variants in the *CFH/CFHR* locus influence systemic complement activation. Genetic and nongenetic factors identified in this and other studies explain up to 18.7% of the variability in systemic complement activation levels. The common variant rs6685931 in *CFHR4*, and its associated haplotype H1−2, could be used, together with other environmental factors as well as rare genetic variants, to select AMD patients who would benefit from complement-inhibiting therapies.

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References

1. Ricklin D, Hajishengallis G, Yang K, et al. Complement: a key system for immune surveillance and homeostasis. *Nat Immunol*. 2010;11(9):785−797.
2. Dunkelberger JR, Song WC. Complement and its role in innate and adaptive immune responses. *Cell Res*. 2010;20(1):34−50.
3. Lachmann PJ, Halbwachs L. The influence of C3b inactivator (KAF) concentration on the ability of serum to support complement activation. *Clin Exp Immunol*. 1975;21(1):109−114.
4. Sarma JV, Ward PA. The complement system. *Cell Tissue Res*. 2011;343(1):227−235.
5. McGeer PL, Lee M, McGeer EG. A review of human diseases caused or exacerbated by aberrant complement activation. *Neurobiol Aging*. 2017;52:12−22.
6. Rudnicka AR, Jarrar Z, Wormald R, et al. Age and gender variations in age-related macular degeneration prevalence in populations of European ancestry: a meta-analysis. *Ophthalmology*. 2012;119(3):571−580.
7. Chakravarthy U, Evans J, Rosenfeld PJ. Age related macular degeneration. *BMJ (Clin Res ed.)*. 2010;340:c981.
8. Wong WL, Su X, Li X, et al. Global prevalence of age-related macular degeneration and disease burden projection for 2020 and 2040: a systematic review and meta-analysis. *Lancet Glob Health*. 2014;2(2):e106−e116.
9. Fritsche LG, Igl W, Bailey JN, et al. A large genome-wide association study of age-related macular degeneration highlights contributions of rare and common variants. *Nat Genet*. 2016;48(2):134−143.
10. Geerlings MJ, de Jong EK, den Hollander AI. The complement system in age-related macular degeneration: A review of rare genetic variants and implications for personalized treatment. *Mol Immunol*. 2017;84:65−76.
11. Hageman GS, Luthert PJ, Victor Chong NH, et al. An integrated hypothesis that considers drusen as biomarkers of immune-mediated processes at the RPE-Bruch's membrane interface in aging and age-related macular degeneration. *Prog Retin Eye Res*. 2001;20(6):705−732.

12. Johnson LV, Leitner WP, Staples MK, et al. Complement activation and inflammatory processes in Drusen formation and age related macular degeneration. *Exp Eye Res*. 2001;73(6):887−896.
13. Wang L, Clark ME, Crossman DK, et al. Abundant lipid and protein components of drusen. *PLoS One*. 2010;5(4):e10329.
14. Fernandez-Godino R, Garland DL, Pierce EA. A local complement response by RPE causes early-stage macular degeneration. *Hum Mol Genet*. 2015;24(19):5555−5569.
15. Sivaprasad S, Adewoyin T, Bailey TA, et al. Estimation of systemic complement C3 activity in age-related macular degeneration. *Arch Ophthalmol*. 2007;125(4):515−519.
16. Scholl HP, Charbel Issa P, Walier M, et al. Systemic complement activation in age-related macular degeneration. *PLoS One*. 2008;3(7):e2593.
17. Reynolds R, Hartnett ME, Atkinson JP, et al. Plasma complement components and activation fragments: associations with age-related macular degeneration genotypes and phenotypes. *Invest Ophthalmol Vis Sci*. 2009;50(12):5818−5827.
18. Hecker LA, Edwards AO, Ryu E, et al. Genetic control of the alternative pathway of complement in humans and age-related macular degeneration. *Hum Mol Genet*. 2010;19(1):209−215.
19. Smailhodzic D, Klaver CC, Klevering BJ, et al. Risk alleles in *CFH* and *ARMS2* are independently associated with systemic complement activation in age-related macular degeneration. *Ophthalmology*. 2012;119(2):339−346.
20. Lechner J, Chen M, Hogg RE, et al. Higher plasma levels of complement C3a, C4a and C5a increase the risk of subretinal fibrosis in neovascular age-related macular degeneration: complement activation in AMD. *Immun Ageing*. 2016;13:4.
21. Ristau T, Paun C, Ersoy L, et al. Impact of the common genetic associations of age-related macular degeneration upon systemic complement component C3d levels. *PLoS One*. 2014;9(3):e93459.
22. Smailhodzic D, van Asten F, Blom AM, et al. Zinc supplementation inhibits complement activation in age-related macular degeneration. *PLoS One*. 2014;9(11):e112682.
23. Yehoshua Z, de Amorim Garcia Filho CA, Nunes RP, et al. Systemic complement inhibition with eculizumab for geographic atrophy in age-related macular degeneration: the COMPLETE study. *Ophthalmology*. 2014;121(3):693−701.
24. Xu H, Chen M. Targeting the complement system for the management of retinal inflammatory and degenerative diseases. *Eur J Pharmacol*. 2016;787:94−104.
25. Paun CC, Lechanteur YT, Groenewoud JM, et al. A novel complotype combination associates with age-related macular degeneration and high complement activation levels in vivo. *Sci Rep*. 2016;6:26568.
26. Rother E, Lang B, Coldewey R, et al. Complement split product C3d as an indicator of disease activity in systemic lupus erythematosus. *Clin Rheumatol*. 1993;12(1):31−35.
27. Michel O, Sergysels R, Duchateau J. Complement activation in bronchial asthma evaluated by the C3d/C3 index. *Ann Allergy*. 1986;57(6):405−408.
28. Galle C, De Maertelaer V, Motte S, et al. Early inflammatory response after elective abdominal aortic aneurysm repair: a comparison between endovascular procedure and conventional surgery. *J Vasc Surg*. 2000;32(2):234−246.
29. Hempel JC, Poppelaars F, Gaya da Costa M, et al. Distinct in vitro complement activation by various intravenous iron preparations. *Am J Nephrol*. 2017;45(1):49−59.
30. Purcell S, Cherny SS, Sham PC. Genetic power calculator: design of linkage and association genetic mapping studies of complex traits. *Bioinformatics*. 2003;19(1):149−150.
31. Pruim RJ, Welch RP, Sanna S, et al. LocusZoom: regional visualization of genome-wide association scan results. *Bioinformatics*. 2010;26(18):2336−2337.
32. Willer CJ, Li Y, Abecasis GR. METAL: fast and efficient meta-analysis of genomewide association scans. *Bioinformatics*. 2010;26(17):2190−2191.
33. Paun CC, Ersoy L, Schick T, et al. Genetic variants and systemic complement activation levels are associated with serum lipoprotein levels in age-related macular degeneration. *Invest Ophthalmol Vis Sci*. 2015;56(13):7766−7773.
34. Hageman GS, Anderson DH, Johnson LV, et al. A common haplotype in the complement regulatory gene factor H (HF1/CFH) predisposes individuals to age-related macular degeneration. *Proc Natl Acad Sci U S A*. 2005;102(20):7227−7232.
35. Rodriguez de Cordoba S, Esparza-Gordillo J, Goicoechea de Jorge E, et al. The human complement factor H: functional roles, genetic variations and disease associations. *Mol Immunol*. 2004;41(4):355−367.
36. Chen M, Forrester JV, Xu H. Synthesis of complement factor H by retinal pigment epithelial cells is down-regulated by oxidized photoreceptor outer segments. *Exp Eye Res*. 2007;84(4):635−645.
37. Brooimans RA, van der Ark AA, Buurman WA, et al. Differential regulation of complement factor H and C3 production in human umbilical vein endothelial cells by IFN-gamma and IL-1. *J Immunol*. 1990;144(10):3835−3840.
38. Caprioli J, Castelletti F, Bucchioni S, et al. Complement factor H mutations and gene polymorphisms in haemolytic uraemic syndrome: the C-257T, the A2089G and the G2881T polymorphisms are strongly associated with the disease. *Hum Mol Genet*. 2003;12(24):3385−3395.
39. Fremeaux-Bacchi V, Kemp EJ, Goodship JA, et al. The development of atypical haemolytic-uraemic syndrome is influenced by susceptibility factors in factor H and membrane cofactor protein: evidence from two independent cohorts. *J Med Genet*. 2005;42(11):852−856.
40. Kugelberg E, Gollan B, Tang CM. Mechanisms in Neisseria meningitidis for resistance against complement-mediated killing. *Vaccine*. 2008;26(suppl 8):I34−I39.
41. Martinon-Torres F, Png E, Khor CC, et al. Natural resistance to meningococcal disease related to CFH loci: meta-analysis of genome-wide association studies. *Sci Rep*. 2016;6:35842.
42. Hebecker M, Jozsi M. Factor H-related protein 4 activates complement by serving as a platform for the assembly of alternative pathway C3 convertase via its interaction with C3b protein. *J Biol Chem*. 2012;287(23):19528−19536.
43. van de Ven JP, Nilsson SC, Tan PL, et al. A functional variant in the CFI gene confers a high risk of age-related macular degeneration. *Nat Genet*. 2013;45(7):813−817.
44. Pickering MC, de Jorge EG, Martinez-Barricarte R, et al. Spontaneous hemolytic uremic syndrome triggered by complement factor H lacking surface recognition domains. *J Exp Med*. 2007;204(6):1249−1256.
45. Schick T, Steinhauer M, Aslanidis A, et al. Local complement activation in aqueous humor in patients with age-related macular degeneration. *Eye (Lond)*. 2017;31(5):810−813.
46. Buttner-Mainik A, Parsons J, Jerome H, et al. Production of biologically active recombinant human factor H in Physcomitrella. *Plant Biotechnol J*. 2011;9(3):373−383.
47. Schmidt CQ, Slingsby FC, Richards A, et al. Production of biologically active complement factor H in therapeutically useful quantities. *Protein Expr Purif*. 2011;76(2):254−263.
48. Ricklin D, Lambris JD. New milestones ahead in complement-targeted therapy. *Semin Immunol*. 2016;28(3):208−222.

49. Ferreira VP, Pangburn MK, Cortes C. Complement control protein factor H: the good, the bad, and the inadequate. *Mol Immunol*. 2010;47(13):2187–2197.
50. Wilczek E, Rzepko R, Nowis D, et al. The possible role of factor H in colon cancer resistance to complement attack. *Int J Cancer*. 2008;122(9):2030–2037.
51. Junnikkala S, Hakulinen J, Jarva H, et al. Secretion of soluble complement inhibitors factor H and factor H-like protein (FHL-1) by ovarian tumour cells. *Br J Cancer*. 2002;87(10):1119–1127.
52. Ajona D, Castano Z, Garayoa M, et al. Expression of complement factor H by lung cancer cells: effects on the activation of the alternative pathway of complement. *Cancer Res*. 2004;64(17):6310–6318.
53. Pastor AF, Rodrigues Moura L, Neto JW, et al. Complement factor H gene (CFH) polymorphisms C-257T, G257A and haplotypes are associated with protection against severe dengue phenotype, possible related with high CFH expression. *Hum Immunol*. 2013;74(9):1225–1230.

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Abbreviations and Acronyms:
AMD = age-related macular degeneration; **AP** = alternative pathway; **BMI** = body mass index; **C3** = component 3; **GWAS** = genome-wide association study; **SE** = standard error; **SNP** = single nucleotide polymorphism.

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